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Differential Regulation of Expression of the Multiple ADP/ATP Translocase Genes in Human Cells*

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The expression of the genes encoding the three isoforms of the human ADP/ATP translocase (T1, T2, and T3) has been investigated in cultured cell systems under different experimental conditions, using isoform-specific probes. In several human cell lines tested, *i.e.* HeLa, Hep3B, 143B, HL60, the T3 gene is expressed as a single 1300-nucleotide mRNA, whereas the T2 gene produces two species of mRNA, 1450 and 1600 nucleotides in size. These two species, which are present in HeLa cells in approximately equivalent amounts, were shown to derive from the use of two different polyadenylation signals. The gene for the muscle-specific isoform of ADP/ATP translocase, T1, was not found to be expressed in any of the cell lines investigated. The levels of T2 and T3 mRNAs in HeLa cells are differentially affected by the growth conditions. In fact, the T2 mRNA level remains relatively constant throughout the exponential and stationary phases, whereas the T3 mRNA level decreases progressively in the second half of the exponential phase and in the stationary phase down to less than 50%. This difference in quantitative behavior of the two mRNAs must reflect changes in their rates of synthesis, since their half-lives are very similar ($t_{1/2}$ = 5–6 h), with no significant growth-related differences. Treatment of HL60 cells with 12-*O*-tetradecanoylphorbol-13-acetate or retinoic acid, two agents which induce cessation of cell proliferation and cell differentiation, resulted in a marked decrease in both T2 and T3 mRNA levels. Exposure of HeLa cells to chloramphenicol produced a pronounced decrease in the levels of both T2 and T3 mRNAs after 48 to 72 h of treatment. Half-life time measurements strongly suggested that this decrease reflected a reduction in the rate of synthesis of the two transcripts. Treatment of HeLa cells with dinitrophenol also produced a dramatic decrease in the steady state levels of both T2 and T3 mRNA, which, however, in contrast to the just mentioned situation, could be accounted for by a decrease in their metabolic stability. Control experiments indicated that the chloramphenicol- and dinitrophenol-induced changes were not a nonspecific consequence of mitochondrial dysfunction. The observations reported here clearly demonstrate that the expression of the multiple ADP/ATP translocase genes in human cells is sensitive to the cell physiological conditions, responding to the varying cellular demands by changes in the rate of synthesis or stability of their mRNAs.

The adenine nucleotide translocase is a nuclear-encoded integral protein of the inner mitochondrial membrane (for reviews, see Vignais *et al.*, 1985; Klingenberg, 1989). This enzyme, which is the most abundant protein in mitochondria, exchanges ADP with ATP between the cytosol and the mitochondrial matrix in an electrogenic process (Laris, 1977). Such exchange, which links the compartment of ATP synthesis to those of ATP utilization, is essential for the aerobic energy metabolism of the cell. On the basis of amino acid sequence similarities observed with other mitochondrial translocators, the ADP/ATP translocase appears to be a member of a large mitochondrial carrier family. This includes the uncoupler binding protein (for review, see Klingenberg, 1990), the phosphate carrier (Runswick *et al.*, 1987), and the dicarboxylate and α-ketoglutarate-malate carriers (Bisaccia *et al.*, 1988).

Because of its central role in the cell energy metabolism, and because mitochondrial energy production varies among tissues, it is likely that the expression of the ADP/ATP translocase gene(s) is regulated in response to functional and developmental factors. The first evidence of tissue-specificity of the bovine ADP/ATP translocase came from an analysis of the immunological reactivity of the translocase extracted from different tissues (Schultheiss and Klingenberg, 1984). The existence of such isoforms has been confirmed in man using a different approach. In fact, the occurrence of at least three different genes, T1, T2, and T3, coding for the human ADP/ATP translocase has been shown (Battini *et al.*, 1987; Neckelmann *et al.*, 1987; Houldsworth and Attardi, 1988; Cozens *et al.*, 1989; Li *et al.*, 1989; Ku *et al.*, 1990). The existence of multiple nuclear genes for the ADP/ATP translocase has also been reported in cow (Powell *et al.*, 1989), *Saccharomyces cerevisiae* (Lawson and Douglas, 1988; Kolarov *et al.*, 1990) and *Zea mays* (Bathgate *et al.*, 1989).

The results reported in this paper demonstrate changes in the expression of the T2 and T3 genes in cultured human cells under conditions which are expected to affect the cellular energy metabolism. In particular, changes in the levels or metabolic stabilities of the T2- and T3-specific mRNAs have been observed in response to growth conditions, differentiation state of the cells, and mitochondrial inhibitors.

EXPERIMENTAL PROCEDURES

Cell Lines—HeLa S3 cells were grown in suspension as described (Amaldi and Attardi, 1968). HL60 cells (Collins *et al.*, 1977), grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal

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calf serum, were induced to differentiate by addition of 52 nM TPA[1] or 1.25 mM RA. Other cell lines used in this work include Hep 3B, derived from a human hepatocellular carcinoma (American Tissue Culture Collection CRL HB8064), and 143B, derived from a human osteosarcoma (ATCC CRL 8303).

RNA Isolation and Gel Blot Analysis—Total cellular RNA from tissues and various cell lines was extracted using guanidinium isothiocyanate (Chirgwin *et al.*, 1979). Polyadenylated RNA was isolated by double passage over an oligo(dT)-cellulose column (Amalric *et al.*, 1978) and fractionated by electrophoresis through a 1.4% agarose, 2.2 M formaldehyde gel in MOPS buffer (Davis *et al.*, 1986). The RNA integrity in the samples before oligo(dT)-cellulose chromatography fractionation was verified by running a portion of each sample on gel and checking the ratio of the two cytosolic rRNAs after ethidium bromide staining. RNA was transferred from the gels onto Zeta-Probe membranes (Bio-Rad) by electroblotting. For quantitative analysis, total RNA samples were applied by filtration onto a Zeta-Probe membrane using a Schleicher & Schuell Minifold II slot blotter apparatus. RNA on the membranes was fixed by baking for 2 h at 80 °C in a vacuum oven, and the membranes were then washed, pretreated, and incubated for hybridization as described (Houldsworth and Attardi, 1988), with some slight modifications. In particular, hybridization of cDNA-derived probes was carried out for 18 h at 42 °C in the presence of 40% formamide (FA) and 5 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0), hybridization of oligonucleotide probes for 18 h at 52 °C, in the absence of FA and in the presence of 6 × SSC. ^{32}P-Labeled probes were prepared by random priming labeling (Feinberg and Vogelstein, 1983; Cobianchi and Wilson, 1987) (T2 and T3 cDNA clone inserts, GPDHase cDNA) or filling-in (T1 cDNA clone insert) to a specific activity of 5–6 × 10^8 cpm/mg, or by 5′-end labeling of purified synthetic oligonucleotides with T4 polynucleotide kinase and [γ-^{32}P]ATP. After hybridization with the different labeled probes (4 × 10^6 to 2 × 10^7 cpm), the filters were processed as described previously (Houldsworth and Attardi, 1988).

Southern Blot Analysis—Total DNA was extracted from HeLa cells by the method of Hermann and Frischauf (1987) and digested with restriction endonucleases in the buffers recommended by the suppliers. The digested DNA was fractionated on a 0.8% agarose gel in 50 mM Tris-borate, 1 mM EDTA, and, after denaturation in 0.5 M NaOH, 1.5 M NaCl, it was transferred by capillarity onto a Zeta-Probe membrane in 1 M ammonium acetate, 20 mM NaOH overnight (Southern, 1975; Rigaud *et al.*, 1987). Hybridization of the DNA to the different labeled cDNA probes (1.0–1.7 × 10^7 cpm) was carried out at 42 °C in 40% FA, 5 × SSC, 0.1% sodium dodecyl sulfate, 1 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 20 mM sodium pyrophosphate, 10% dextran sulfate, and 0.01% salmon sperm DNA.

DNA Probes—A specific cDNA probe for T1 was subcloned by digestion of the clone pHAT 14 (Houldsworth and Attardi, 1988) with *Nci*I (Fig. 1), filling-in the ends of the fragment, and ligating it into the *Hin*dII site of the polylinker of pUC9. After digestion of the recombinant pUC9 derivative with *Bam*HI and *Eco*RI, the insert was isolated by electrophoresis in a sodium dodecyl sulfate-polyacrylamide gel. T2- and T3-specific cDNA probes were obtained by digestion, respectively, of the clone pHAT 8 with *Ban*II and of the clone pHAT 3 with *Hin*dIII + *Mae*III, and isolation of the fragments by agarose gel electrophoresis. Two oligonucleotides complementary to the coding strand of the T1 gene were synthesized: T1–1 (nt 1204–1228) and T1–2 (nt 3–27). The T1–1 probe exhibits about 13% sequence similarity to the corresponding T2 and T3 segments, whereas the T1–2 probe has 42% and, respectively, 50% similarity to the corresponding T2 and T3 sequences. The nucleotide numbering in the cDNA probes and oligonucleotides used in the present work corresponds to that used for the cDNA sequences, as previously reported (Houldsworth and Attardi, 1988). The glyceraldehyde-3-phosphate dehydrogenase (GPDHase) probe was a full-length rat cDNA clone (Fort *et al.*, 1985) (kindly provided by Dr. Ph. Jeanteur).

RESULTS

ADP/ATP Translocase Gene-specific Probes—To facilitate a comparison between the various data in the literature, we will use the following terminology: T1 will be used to refer to the ANT1 gene (Li *et al.*, 1989), the T1 gene (Cozens *et al.*, 1989) and the pHAT14 partial cDNA clone (Houldsworth and Attardi, 1988). T2 will be used to refer to the T2 gene (Cozens *et al.*, 1989) and to the pHAT8 partial cDNA clone (Houldsworth and Attardi, 1988). T3 will be used to refer to the hp2F1 cDNA clone (Battini *et al.*, 1987), designated as ANT2 by other authors (Li *et al.*, 1989), and to the pHAT3 partial cDNA clone (Houldsworth and Attardi, 1988). In order to achieve a high selectivity in the analysis of the HeLa cells mRNAs and in the Southern blot analysis, three cDNA probes corresponding to segments of the pHAT14, pHAT8, and pHAT3 partial cDNA clones (Houldsworth and Attardi, 1988) were isolated. The specific probes, shown in Fig. 1, were characterized by a relatively low degree of nucleotide sequence similarity (see legend). Specific oligonucleotides corresponding to selected regions of the cDNAs were also synthesized (see below).

Number of Genes for the ADP/ATP Translocase—Southern blot hybridization experiments were carried out on digests of total HeLa cell DNA obtained with three restriction enzymes (*Eco*RI, *Bam*HI, and *Hin*dIII). On the basis of the published cDNA sequences (Battini *et al.*, 1987; Neckelmann *et al.*, 1987; Cozens *et al.*, 1989), these enzymes were not expected to cut the portions of the cDNA sequences used as probes. Furthermore, on the basis of the genomic sequences (Cozens *et al.*, 1989; Ku *et al.*, 1990), the various cDNA probes did not overlap adjacent exons. As shown in Fig. 2, in all digests, the presence of only one band reacting with a T1-specific probe and of at least two bands reacting with a T2-specific probe was detected. On the other hand, experiments conducted with a specific probe derived from the T3 cDNA revealed a much greater complexity of the Southern blot hybridization pattern. These results are consistent with there being a single gene and no pseudogene for T1, one or two genes for T2, and several genes for T3, possibly including pseudogenes.

Identification of the mRNA Species Expressed in HeLa Cells—A previous analysis of the ADP/ATP translocase mRNAs expressed in HeLa cells had revealed three discrete mRNA species, with sizes of 1300, 1450 and 1600 nt, respectively (Houldsworth and Attardi, 1988). The authors reported that the T2 cDNA probe used (pHAT8) reacted with all three

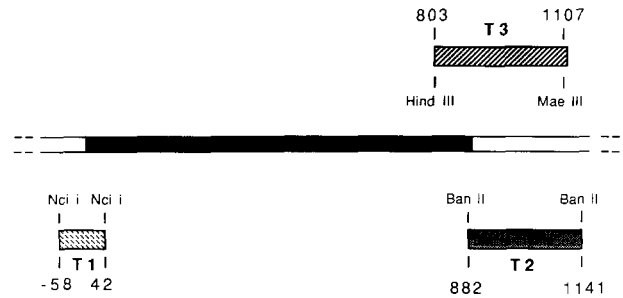

FIG. 1. **Isoform-specific probes derived from the ADP/ATP translocase cDNAs.** The probes were isolated as described under "Experimental Procedures." *Probe T1*, which is 124 nt long, contains a 101-nt fragment (−58 to +42) of the cDNA generated by *Nci*I digestion. This fragment overlaps a portion of the 5′-noncoding region and the first 42 coding nucleotides in exon 1 of the T1 gene, and shows 51% and, respectively, 52% sequence similarity to the corresponding segments of T2 and T3. *Probe T2*, which is 259 nt long, overlaps the last 13 coding nucleotides and a portion of the 3′-noncoding sequence in exon 4 of the T2 gene, and shows 16% and, respectively, 14% sequence similarity to the corresponding stretches of T1 and T3. *Probe T3*, which is 304 nt long, overlaps the last 91 coding nucleotides and a portion of the 3′-noncoding sequence of the T3 gene, and shows 41% and, respectively, 39% sequence similarity to the corresponding segments of T1 and T2.

[1] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; RA, retinoic acid; GPDHase, glyceraldehyde-3-phosphate-dehydrogenase; CAP, chloramphenicol; FA, formamide; nt, nucleotide; MOPS, 4-morpholinepropanesulfonic acid.

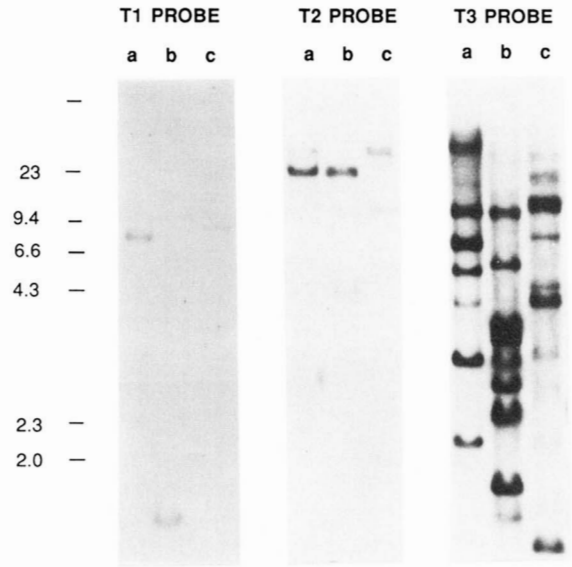

FIG. 2. **Hybridization of digests of human DNA with probes derived from human ADP/ATP translocase cDNAs.** The specific hybridization probes illustrated in Fig. 1 were employed. HeLa S3 total DNA was digested with the following restriction endonucleases. *a*, *Bam*HI; *b*, *Eco*RI; *c*, *Hin*dIII. The digests were fractionated in a 0.8% agarose gel, transferred onto a Zeta-Probe membrane, and hybridized with the specific ^{32}P-labeled probes, as detailed under "Experimental Procedures." Size markers correspond to fragments of bacteriophage lambda DNA generated by *Hin*dIII digestion.

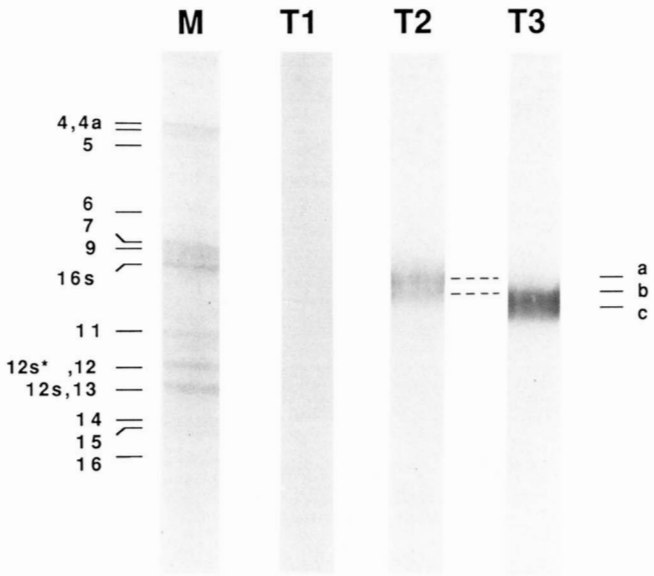

FIG. 3. **RNA transfer hybridization analysis of HeLa cell mRNA.** Polyadenylated mRNA was isolated from exponentially growing HeLa cells, fractionated by electrophoresis in a 1.4% agarose, 2.2 M formaldehyde gel (4 μg/lane), electroblotted onto a Zeta-Probe membrane, and hybridized with the specific ^{32}P-labeled probes, as described under "Experimental Procedures" (*T1*, 5×10^6 cpm, 36 h exposure; *T2*, 4×10^6 cpm, 24 h exposure; *T3*, 6×10^6 cpm, 24 h exposure). Mitochondrial RNA species labeled in isolated organelles (Gaines and Attardi, 1984) were used as molecular weight markers (*lane M*). The sizes of 16 S rRNA, RNA 11, 12 S* rRNA, and 12 S rRNA are 1559, 1200, 1045, and 954 nt, respectively.

species, the most strongly hybridizing species being the 1450-nt mRNA. The T1 cDNA probe (pHAT14) hybridized only very weakly with the 1300-nt mRNA species; on the contrary the T3 cDNA probe (pHAT3) gave a strong signal with this mRNA, and only a marginal reaction with the other two mRNA species. The cross-hybridization observed between the three mRNA species resulted from the high nucleotide sequence similarity existing between the T1, T2, and T3 sequences, especially in the protein coding sequence (Cozens *et al.*, 1989).

An RNA transfer hybridization analysis of HeLa cell mRNA carried out under the conditions described under "Experimental Procedures" showed no hybridization with the T1-specific probe (Fig. 3). This also held true under conditions of hybridization and washing of much lower stringency. Similarly, no hybridization of the T1 probes (cDNA fragment and T1–1 and T1–2 oligonucleotides) was detected with the mRNA from the other cell lines tested, *i.e.* Hep 3B, 143B, and HL60. In all human cell lines used in this work, the specific T2 probe hybridized strongly with the two larger mRNA species (1450 and 1600 nt), whereas the T3-specific probe reacted only with the shorter mRNA species (1300 nt).

Two Polyadenylation Signals Are Used during Transcription of the T2 Gene in HeLa Cells—In their analysis of the sequence of the T2 gene, Cozens *et al.* (1989) described the presence of a typical polyadenylation signal AATAAA located 428 nt after the stop codon and 15 nt upstream of the polyadenylation site in the homologous bovine gene. On the other hand, Houldsworth and Attardi (1988) reported the presence of a poly(A) tail starting 326 nt after the stop codon in their cDNA clone corresponding to the T2 gene (pHAT8). No typical polyadenylation signal was found upstream of this poly(A) tail. To investigate the possibility that the 1450- and 1600-nt mRNAs hybridizable with the T2 probe reflected the processing of the mRNA precursors at the two polyadenylation sites identified by the above cited authors, two oligonucleotides complementary to mRNA sequences located, respectively, 21-nt 5′ to the upstream poly(A) tail (T2–1: 5′ CACGACTTGGCTCCTACAAGCA), and 15-nt 5′ to the canonical AATAAA signal (T2–2: 5′ GCCCAACATAGAACATACTC), were synthesized (Fig. 4). RNA transfer hybridization analysis of HeLa cell mRNA was performed using the two oligonucleotides as probes. Fig. 4 shows clearly that only T2–1 probe was able to hybridize with the two mRNA species, whereas T2–2 probe could only detect the longer mRNA species. Similar results were obtained with the mRNA from other cellular systems, in particular the Hep 3B, 143B, and HL60 cell lines and cultured human myoblasts (not shown). These results strongly suggest that the two mRNA species derive indeed from the use of the canonical AATAAA signal and of an alternate nontypical signal upstream of nt 326, possibly ATTTAAA (Swimmer and Shenk, 1985). When cells were grown under normal conditions, both polyadenylation signals appeared to be used with the same efficiency, as judged from the results of the RNA transfer hybridization experiments. On the contrary, when HeLa cells were grown in the presence of 0.2 mM oligomycin, an inhibitor of the mitochondrial H^+-ATPase, the steady state amount of the shorter mRNA species was significantly higher than that of the species using the typical polyadenylation signal. The physiological significance of this observation remains, however, unknown.

The T2 and T3 Genes Are Differentially Expressed at Different Phases of HeLa Cell Growth—The T3 gene expressed in HL60 cells has been reported to be a growth-regulated gene (Battini *et al.*, 1987). However, in the just cited work, a whole T3 cDNA probe, not apt to distinguish between mRNAs for different isoforms of the translocase, was used. In the present work, the hypothesis that synthesis or stability of the different ADP/ATP translocase mRNAs expressed in HeLa cells may depend upon the growth-phase was tested. The level of the different mRNAs was estimated either after transfer hybridization or after slot-blot hybridization of the total RNA sam-

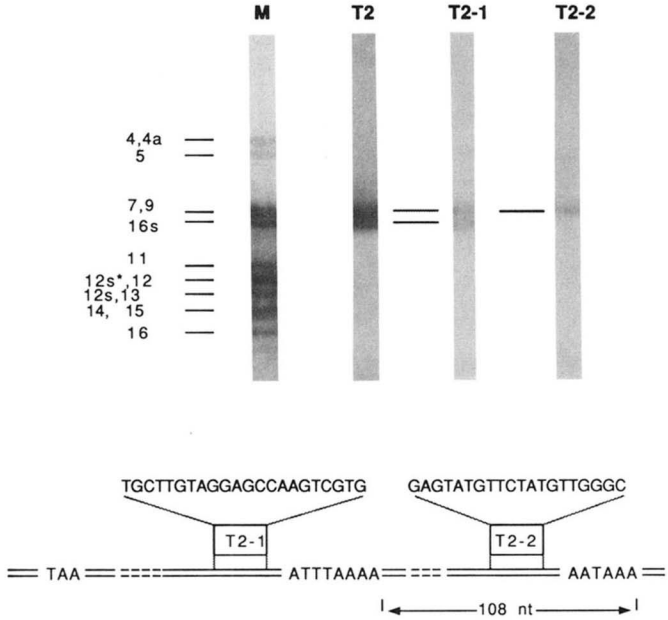

FIG. 4. **RNA transfer hybridization analysis of the T2-specific mRNAs.** Total RNA (12 μg) from exponentially growing HeLa cells was fractionated on a formaldehyde/agarose gel and electroblotted onto a Zeta-Probe membrane. The filters were incubated with the ^{32}P-labeled T2-specific cDNA probe and the ^{32}P-labeled T2-specific oligonucleotides T2–1 and T2–2, complementary to the indicated sequences, as detailed under "Experimental Procedures" (*T2*, 4×10^6 cpm, 36 h exposure; *T2–1*, 2×10^7 cpm, 48 h exposure; *T2–2*, 1.7×10^7 cpm, 48 h exposure). *M*, HeLa cell mitochondrial RNAs labeled in isolated organelles (see Fig. 3).

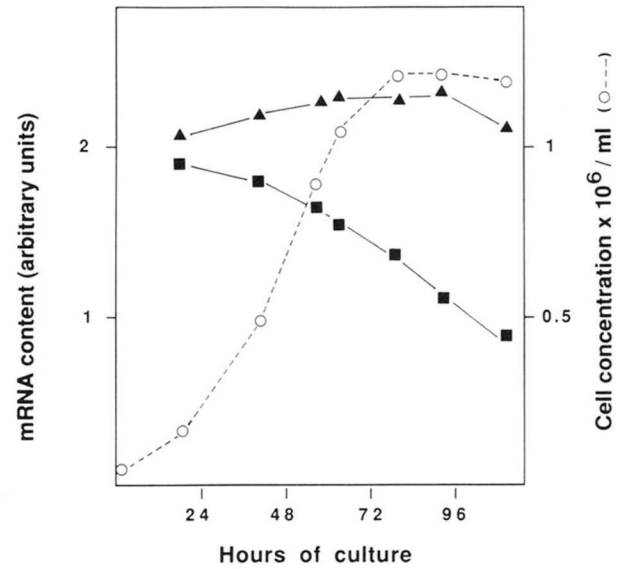

FIG. 5. **Analysis of the ADP/ATP translocase mRNA species expressed at different stages of HeLa cell growth.** Samples (12 μg) of total RNA extracted from HeLa cell suspensions harvested at the indicated times were applied by filtration onto Zeta-Probe membranes, using a slot-blotter apparatus. The membranes were incubated with the ^{32}P-labeled T2-specific (▲) and T3-specific (■) cDNA-derived probes, as described under "Experimental Procedures." The mRNAs were quantitated by densitometric scanning of the autoradiograms. Cell growth was estimated by measuring the cell concentration in a parallel culture at the indicated times (○). An RNA transfer hybridization analysis of the total RNA samples, performed with T1-, T2-, and T3-specific probes, showed lack of any sign of degradation of the T2 and T3 translocase mRNAs and absence of the T1 mRNA.

ples extracted from HeLa cells harvested at different stages of growth. Fig. 5 shows that the level of the T2 mRNA remained relatively constant throughout the exponential and the early and late stationary phases. By contrast, the level of the T3 mRNA decreased as the cell culture approached and reached the stationary phase, down to less than 50%. A significant decrease was already observed in the late portion of the exponential phase. Ethidium bromide staining of the different RNA preparations indicated that the differences in the ADP/ATP translocase mRNA levels were not the consequence of a differential degradation of the RNA samples.

To address the possibility that the level of expression of the T2 and T3 genes is regulated at a posttranscriptional level, the stability of T2 and T3 transcripts in the exponential and stationary phases of growth was investigated. For this purpose, DNA transcription was blocked with actinomycin D in HeLa cells at different stages of growth, and the amount of mRNA surviving after various times of drug treatment was measured by hybridizing cDNA probes labeled by random priming to fixed amounts of total cell RNA slot-blotted onto a Zeta-Probe membrane. As shown in Table I, the T2 and T3 gene-specific transcripts have almost identical half-lives (respectively, 6 and 5 h), pointing to a regulation of expression of these genes at the transcriptional level. Furthermore, for both genes, no significant difference in the half-life of the mRNAs extracted from cells in the exponential and stationary phases of growth could be detected.

T2 and T3 Gene Expression Is Affected by the Differentiation State of HL60 Cells—HL60 cells are a human promyelocytic leukemia cell line in which the promyelocyte can be induced to differentiate by treatment with various agents (for review, Koeffer, 1983). TPA induces terminal differentiation in HL60 along the monocyte-macrophage pathway, whereas RA induces differentiation along the granulocyte pathway. In a

TABLE I

Half-lives of the ADP/ATP translocase T2 and T3 mRNAs at different stages of HeLa cell growth and in the presence of CAP and DNP

Exponentially growing HeLa cells (6×10^5 cells/ml), stationary phase cells (1.2×10^6 cells/ml) and cells grown in presence of CAP or DNP, as indicated, were treated with 5 μg/ml actinomycin D. After various times of exposure to the latter drug, cells were collected, total RNA was extracted, and identical amounts (12 μg) of RNA from the different samples were subjected to slot blot hybridization, as described under "Experimental Procedures." Zero time of actinomycin D treatment was used as a reference for the initial mRNA content.

Growth conditions	$t_{1/2}$	
	T2	T3
	h	
No drug		
Exponential phase	6	5
Stationary phase	6	5
CAP 40 μg/ml, 3 days	6	6.5
DNP 200 μM, 12 h	1.5	2

previously cited work (Battini *et al.*, 1987), a decrease in the level of ADP/ATP translocase mRNA(s) hybridizable with a non-isoform-specific whole T3 cDNA probe was reported in HL60 cells induced to differentiate by either TPA or RA. It seemed interesting to re-examine this phenomenon using isoform-specific probes. Therefore, exponentially growing HL60 cells were treated either with 52 nM TPA or 1.25 mM RA for 72 h. Cytoplasmic RNA was extracted and electrotransferred, and the RNA blots were hybridized with the T1-, T2-, and T3-specific probes. As shown in Fig. 6, both TPA and RA caused a marked decrease in the level of both

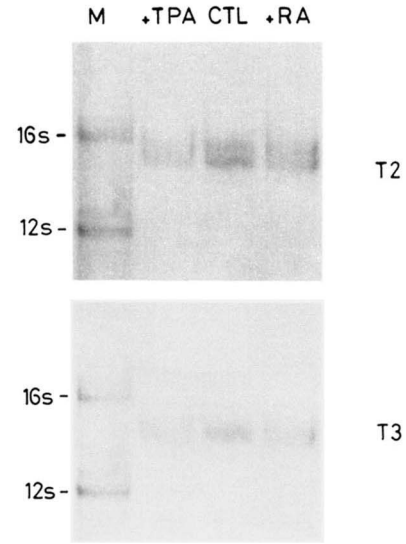

FIG. 6. **Levels of ADP/ATP translocase mRNAs in HL60 cells after treatment with TPA and RA.** Suspensions of exponentially growing HL60 cells (4×10^5/ml) were either treated with 52 nM TPA or with 1.25 mM RA, or maintained untreated as a control (*CTL*). The cells were harvested after 72 h, total RNA was extracted, and 11 μg samples were run on an agarose/formaldehyde gel, transferred onto Zeta-Probe membranes and hybridized with ^{32}P-labeled T2-specific and T3-specific cDNA-derived probes, as described under "Experimental Procedures." *M*, mitochondrial RNA species labeled in isolated organelles (see Fig. 3).

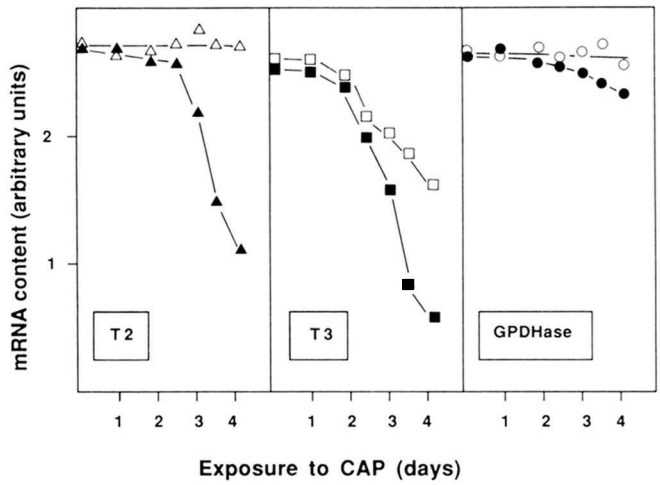

FIG. 7. **Effect of CAP on the expression of the ADP/ATP translocase genes in HeLa cells.** 400 ml of HeLa cell suspension cultures (2×10^5 cells/ml) were exposed to 40 μg/ml CAP and harvested at the indicated times. Parallel HeLa cell cultures, grown under the same conditions except for the absence of CAP, were used as controls. Samples (12 μg) of total RNA extracted from the various cultures were slot-blotted onto Zeta-Probe membranes, and the membranes were then incubated with a ^{32}P-labeled T2-specific (△, ▲), T3-specific (□, ■) or GPDHase-specific (○, ●) cDNA-derived probe, as described under "Experimental Procedures." The mRNAs extracted from CAP-treated cells (▲, ■, ●) and those extracted from control cells (△, □, ○) were quantitated by densitometric scanning of the autoradiograms.

T2 and T3 mRNAs after 72 h of exposure of the cells to these agents. A quantitative analysis showed that the T2 and T3 mRNA levels in TPA-treated cells had decreased to 40% of those in non-treated HL60 cells (CTL), whereas the levels of T2 and T3 mRNAs in RA-treated cells ranged between 55 and 65% of the control levels. No mRNA species corresponding to the T1 gene was detected in HL60 cells under these differentiating conditions.

ADP/ATP Translocase Gene Expression Is Affected by the Physiological State of the Mitochondria—Expression of some nuclear genes encoding mitochondrial proteins is known to be regulated according to the growth conditions and the metabolic demands of the cell (Guarente and Mason, 1983; Szekely and Montgomery, 1984; Parikh *et al.*, 1987; Attardi and Schatz, 1988). Since the ADP/ATP translocase is a protein which plays a major role in the energy metabolism of the cell linked to respiration, the effects of chloramphenicol (CAP) and dinitrophenol (DNP), two drugs which affect, respectively, the biosynthetic and energy yielding processes of the organelles, were tested.

HeLa cells were exposed to a concentration of CAP (40 μg/ml) which is sufficient to inhibit mitochondrial protein synthesis. It was previously found that, under these conditions, cells grew at a normal rate for two doublings, with only a slight decrease in cell size, and then entered a stationary phase, as contrasted to control cells which continued to grow for at least five doublings (Storrie and Attardi, 1972). Cells grown in the presence of CAP were harvested at different times, and the amounts of the different ADP/ATP translocase mRNAs were measured. In Fig. 7, the T2 and T3 mRNA levels in CAP-treated cells are compared to those in control cells grown under the same conditions except for the absence of CAP. In agreement with the observations reported above, in the control cultures, the T2 mRNA level remained approximately constant for more than 4 days, relative to total cell RNA, whereas the T3 mRNA level declined to about 50%. By contrast, in the CAP-treated cells, the amount of T2 and T3 mRNA remained stable for 3 and, respectively, 2 days, and then dropped sharply during the following days. It has previously been shown that CAP, at the concentration used in the present experiments, does not affect nuclear RNA synthesis for at least 3 days (Storrie and Attardi, 1972). Furthermore, in the present work, the total RNA content/cell did not decline after 3 days of CAP treatment relative to the control value, and decreased by only 30% in the fourth day. Therefore, the present data indicate an actual decrease in the levels of T2 and T3 mRNA/cell during CAP treatment. The half-lives of the T2 and T3 mRNAs surviving after 3 days of CAP treatment, determined in the presence of actinomycin D, did not reveal any significant change in the stability of the ADP/ATP mRNAs in the presence of CAP (Table I).

As a control for the specificity of the CAP effects on the T2 and T3 mRNA levels, the relative amounts of mRNA for a non-mitochondrial housekeeping enzyme, GPDHase, were measured in control cells and in CAP-treated cells. To this end, a rat cDNA for GPDHase was used for slot-blot hybridizations, by taking advantage of the high degree of nucleotide sequence conservation found in both coding and 5′- and 3′-noncoding regions between rat and human GPDHase cDNAs (Fort *et al.*, 1985). The rat GPDHase cDNA probe detected in human cell RNA samples an mRNA species ~1400 nt in size, in agreement with previously reported observations (Dani *et al.*, 1984). As shown in Fig. 7, the relative levels of GDPHase remained constant for 4 days in control cells, and declined only very slightly (about 10% after 4 days) in CAP-treated cells.

Exposure of HeLa cells to DNP, a drug which acts as a potent uncoupler of oxidative phosphorylation, altering dramatically the aerobic process of ATP synthesis, resulted in a rapid decrease of the levels of the ADP/ATP translocase mRNAs (Fig. 8). After 24 h of exposure to DNP, the relative amounts of T2 and T3 mRNA were 13% and, respectively, 6% of the original value. The half-lives of T2 and T3 mRNAs,

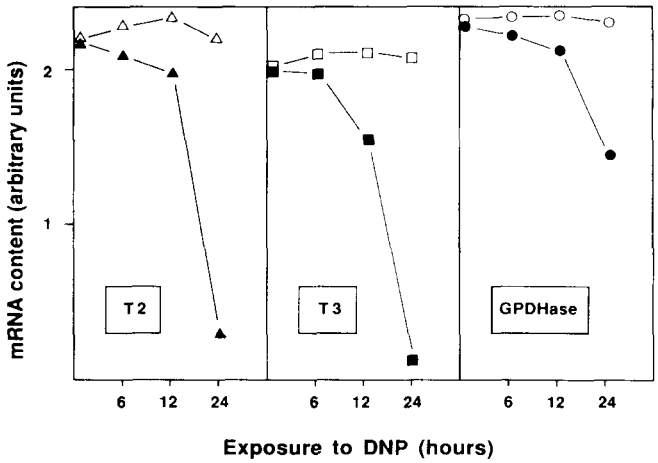

FIG. 8. **Effect of DNP on the expression of the ADP/ATP translocase genes in HeLa cells.** Two HeLa cell suspension cultures (4×10^5 cells/ml) were grown in the presence or absence of 200 mM DNP, and aliquots (200 μl) were harvested at the indicated times. Samples of total RNA extracted from the various aliquots were analyzed as detailed in the legend for Fig. 7. ▲, ■, ●: DNP-treated cells; △, □, ○: control cells grown in the absence of DNP.

estimated in the presence of actinomycin D in cells previously exposed for 12 h to DNP, were much lower than those found in control cells grown under the same conditions except for the absence of DNP (Table I). Fig. 8 shows that, in contrast to the T2 and T3 mRNAs, the GPDHase mRNA was only moderately affected by DNP treatment, its relative level being still 90% of the control value after 12 h and >60% after 24 h.

DISCUSSION

The physiological significance of the existence of tissue-specific isoforms of ADP/ATP translocase is probably related to the different functional properties of these isoforms, which result from their structural differences. It is a plausible hypothesis that differences in the regulatory properties of the ADP/ATP translocase and in its responses to the membrane potential and the ADP/ATP ratio play an essential role in satisfying organ- or cell-specific energetic requirements. Consistent with this hypothesis is the main conclusion of the present study, namely, that the expression of the genes controlling the isoforms of the ADP/ATP translocase is sensitive to the cell metabolic and energetic state and that these genes respond either differentially or coordinately to growth conditions, tissue-specific factors, and metabolic inhibitors.

Hybridization experiments carried out with restriction digests of human DNA using cDNA and oligonucleotide probes highly specific for each of the three translocase cDNAs have indicated the presence in the genome of only one T1-related sequence and two or several T2-related sequences; some of the latter may correspond to pseudogenes (Cozens *et al.*, 1989). Interestingly, hybridization experiments conducted with a T3-specific probe have shown the presence of numerous different sequences related to the T3 cDNA. A possible explanation for the multiplicity of sequences related to T3 in man would be the presence of a family of T3-related genes. It is relevant to mention, in this connection, that, in order to account for some specific metabolic pathways, it has been suggested that an ADP/ATP translocase may be located in other intracellular organelles besides mitochondria (West and Clegg, 1984; Kolarov *et al.*, 1990). On the other hand, the occurrence of several T3-related pseudogenes in the human genome has been recently reported (Chen *et al.*, 1990).

Despite the use of conditions of low stringency hybridization, probes specific for the muscle isoform of the translocase, T1, did not react with the mRNAs extracted from HeLa S3, HL60, Hep 3B, or 143B cells. It should be mentioned, however, that the presence of an mRNA reacting with a T1-specific probe has been reported in HeLa cells (Li *et al.*, 1989). The reason for this discrepancy is not known. However, the possibility that the T1-specific probes failed, under the conditions used here, to hybridize with T1 transcripts could be ruled out, since, in parallel experiments, mRNA extracted from human muscle gave a strong signal with both the oligonucleotide and cDNA T1-specific probes.[2] The muscle-specific T1 mRNA species detected in the last cited experiments had a size of 1400 nt, in agreement with a previous report (Neckelmann *et al.*, 1987) and with gene sequence data (Cozens *et al.*, 1989).

The observation that the steady state level of T3 mRNA decreased progressively during prolonged growth of HeLa cells is consistent with a previous report (Battini *et al.*, 1987) indicating that the T3 mRNA level in Balb/c/3T3 cells is sensitive to growth factors. On the other hand, the relatively constant level of T2 mRNA throughout the exponential and stationary phases of growth points to a regulation of T2 gene expression different from that of the T3 gene. The evidence obtained in the present work indicates that this difference in regulation occurs at the transcriptional level.

The marked decrease in the levels of both T2 and T3 mRNAs observed in HL60 cells treated with TPA or RA is at variance with the phenomenon observed during prolonged culture of HeLa cells, in which the decrease affected mainly the T3 mRNA. In the case of HL60 cells treated with TPA, the decline in the level of the ADP/ATP translocase mRNAs is intriguing, since differentiation along the monocytic-macrophage pathway has been reported to be accompanied by an increase in the amount of the mitochondrial profiles in TPA-treated cells (Rovera *et al.*, 1979).

The results obtained in the present work on the effects of CAP, an inhibitor of mitochondrial protein synthesis, and DNP, a potent uncoupler of mitochondrial oxydative-phosphorylation, clearly illustrate how responsive the expression of the ADP-ATP translocase genes is to the physiological state of mitochondria. Previous studies on the response of nuclear genes encoding mitochondrial proteins to inhibitors of mitochondrial function have revealed differences in behavior of different genes. Thus, treatment of wild-type *Neurospora* cells with CAP has been shown to result in increased synthesis of a number of different mitochondrial constituents encoded by nuclear genes, including mitochondrial ribosomal proteins, cytochrome *c*, alternative oxidase, and possibly mitochondrial RNA polymerase (Kuiper *et al.*, 1988). An increase in the abundance of several nuclear DNA transcripts relative to the wild-type levels has been observed in isochromosomal respiratory-deficient yeast stains, including ρ° petites (Parikh *et al.*, 1987). Similarly, an increase in the expression of the T3 ADP/ATP translocase gene has been detected in the ρ° derivatives of the human 143B cell line, which lack mitochondrial protein synthesis due to the absence of mitochondrial DNA (King and Attardi, 1989).[2] The observations discussed above are consistent with the suggestion that the increase in the level of expression of some nuclear genes encoding mitochondrial proteins may be part of a general cellular response to impaired mitochondrial functions (Kuiper *et al.*, 1988). However, there is also clear evidence that the expression of other nuclear genes for mitochondrial proteins is decreased under the same conditions (Parikh *et al.*, 1987).

The data reported in this paper indicate that the ADP/

[2] J. Lunardi and G. Attardi, unpublished observations.

ATP translocase genes belong to the latter class of genes. In fact, both after CAP or DNP treatment, we observed a decrease in the levels of T2 and T3 mRNAs. A comparison with the behavior of the mRNA for a non-mitochondrial housekeeping enzyme, GPDHase, showed that the observed effects of these inhibitors on T2 and T3 gene expression are not simply the consequence of the cell loss of energy producing capacity, but probably reflect specific phenomena. In the case of the CAP treatment, half-life measurements clearly showed that the decrease in T2 and T3 mRNA levels reflected a reduction in their rates of synthesis. The fact that this reduction occurred only after 2 to 3 days of cell culture in the presence of CAP has possibly the same basis as the delay in inhibition of cell growth observed under the same conditions. Storrie and Attardi (1972) interpreted this delay as reflecting the time required for the pre-existing respiratory capacity of the cell to decrease, due to cessation of assembly of respiratory enzymes, to the point of becoming rate-limiting. Similarly, one could speculate that ADP/ATP translocase gene expression is not affected until the impairing of the mitochondrial respiratory function, resulting from inhibition of the mitochondrial protein synthesis, reaches a certain threshold level.

The observed effects of DNP treatment on translocase gene expression were quite different from those of CAP treatment, since 6–13% of the relative amounts of the ADP/ATP translocase mRNAs present in control cells could be measured in cells treated with 200 mM DNP for 24 h. Furthermore, this decrease in the steady state levels of the translocase mRNAs could well be accounted for by their increased metabolic instability. In the presence of DNP, the inner mitochondrial membrane becomes permeable to H^+, and both the pH gradient and the membrane potential are destroyed. The presence of DNP in the culture medium should block the import of the ADP/ATP translocase into mitochondria, since this process requires a high membrane potential. An intriguing possibility is that the increased instability of the T2 and T3 ADP/ATP translocase mRNAs is related to the accumulation of the protein in the cytosol, reflecting an autoregulation phenomenon similar to that operating in the control of tubulin mRNA stability (Cleveland, 1988).

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